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Voltage-dependent sodium channels and calcium channels have been studied at the single channel level in artificial planar phospholipid bilayer membranes. Sodium channels are responsible for impulse generation in nerve and muscle and constitute the only site of action of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX). The sodium channels retain normal function in the artificial membrane. Membrane depolarization reduced the potency of STX and TTX. Experiments employing well-defined ion composition on both sides of the channel as well as chemical modification of the STX binding site, revealed previous unknown blocking effects of calcium ions on sodium movement through the channels. Calcium channels control the entry of calcium ions into nerve terminals and thus modulate release of neurotransmitters. Calcium channels in planar bilayers display voltage-dependent opening and closing, selectivity for divalent cations and block by calcium channel inhibitors. The probability of channel closing is inversely proportional to the rate of permeant ion movement through the channel, suggesting that only unoccupied channels can cl

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ION CHANNELS FROM MAMMALIAN BRAIN AND HEART, INCORPORATED INTO PLANAR LIPID BILAYERS: REGULATION BY MEMBRANE POTENTIAL, CALCIUM, AND NEUROTOXINS

Annual Summary Report
Period 8/1/83 through 7/31/84

Bruce K. Krueger, Ph.D.
Robert J. French, Ph.D.

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SUMMARY

Sodium channels and calcium channels from rat brain membranes, have been incorporated into planar phospholipid bilayer membranes and characterized electrophysiologically. Currents through single channel molecules (single channel currents) were studied. The sodium channels were activated by the neurotoxin batrachotoxin, and were selective for sodium over potassium, cesium, and chloride. They opened as the membrane was depolarized, and were blocked by nanomolar concentrations of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX). The single channel conductance was 30 pS in symmetrical 0.5 M NaCl, 0.1 mM $CaCl_2$. Block of single sodium channels by STX was found to be dependent on the membrane potential with depolarizing potentials reducing the potency of STX block by as much as 50-fold. Both blocking and unblocking rate constants were affected by the membrane potential: depolarization decreased the rate (probability) of channel block by STX and increased the rate (probability) of unblock. These sodium channels are responsible for depolarizing phase of the action potential in nerve and muscle cells and appear to constitute the sole site of action of STX and TTX. Sodium channels in planar bilayers are blocked by both by external and internal calcium ions. Block by external calcium appears to be competitive with sodium suggesting binding to a common site. Block by external calcium is also voltage-dependent with hyperpolarizing potentials favoring block. Some of the effects of external calcium may be due to binding at the STX binding site since treatment with trimethyloxonium (a carboxyl modifying reagent) eliminates STX block (and binding of ^{3}H-STX), reduces the degree of calcium block, and reduces the single channel conductance by 30%. STX and calcium protect against modification by TMO. Single calcium channels from rat brain membrane vesicles were also incorporated into planar bilayers. The calcium channels were selective for calcium, barium, and strontium over monovalent cations and anions. The single channel conductances for Ca^{++}, Ba^{++}, and Sr^{++} were 5 pS, 8.5 pS, and 5 pS, respectively, in symmetrical 0.25 M $Me^{++}Cl_2$. Membrane depolarization increased the probability of channel opening and decreased the probability of channel closing. There was an apparent reciprocal relationship between the single channel conductance and the mean open lifetime for the three permeant cations tested, suggesting a possible relationship between channel gating (voltage dependence) and ion permeation. These calcium channels may be the pathways for calcium entry during stimulus-coupled release of neurotransmitter at synapses in the central nervous system. Heart sarcolemma membranes have been incorporated into planar bilayers and, in preliminary experiments, STX/TTX-sensitive sodium channels and a novel rectifying cation channel have been identified.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

EXPERIMENTAL RESULTS

A. SPECIFIC AIMS OF LAST PROPOSAL

In this section of the report we state the Specific Aims of our proposal for renewal of the contract beginning 1 February 1984, which was funded for nine months. These specific aims superceded those of the original proposal. Subsequent comments summarize the progress made on each Specific Aim during the second year (8/1/83 - 7/31/84) of the project.

1. To explore [several] aspects of the molecular nature of the interaction of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX) with sodium channels from mammalian central nervous system.

During the first year of the project, we developed the capability to study and characterize voltage-dependent sodium channels incorporated into planar bilayers (Krueger et al., 1983). In the second year we expanded this preliminary study to include the details of toxin block and ion permeation through the channels. In particular both STX and TTX were found to block in a voltage-dependent manner. Sodium permeation through the channels and block of sodium permeation by calcium and strontium ions have been investigated. The results are summarized in sections D and E (French et al., 1984).

2. To describe [several] properties of voltage-dependert calcium channels from rat brain.

We have identified and characterized voltage-dependent calcium channels in planar bilayers exposed to rat brain membrane vesicles. The results are summarized in section F (Nelson et al., 1984).

3. To reconstitute and study sodium channels and calcium channels from mammalian heart sarcolemma in planar bilayers and to compare their properties with those of sodium and calcium channels from brain.

In preliminary experiments, we have identified STX/TTX sensitive sodium channels as well as a novel cation channel in planar bilayers exposed to rat heart sarcolemma membranes (see section G.). To date no evidence for heart calcium channels has been found.

B. PUBLICATIONS AND SCIENTIFIC MEETINGS.

Work under this contract has resulted in the following publications:

Krueger, B.K., J.F. Worley, III, and R.J. French. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. Nature 303: 172-175 (1983).

French, R.J., J.F. Worley, III, and B.K. Krueger. Voltage-dependent block by saxitoxin of sodium channels incorporated into planar lipid bilayers. Biophysical Journal 45: 301-310 (1984).

Nelson, M.T., R.J. French, and B.K. Krueger. Single calcium channels from rat brain in planar lipid bilayers. Nature 308: 77-80 (1984).

Nelson, M.T. Single calcium channels from rat brain in planar lipid bilayers. in "Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects" (F. Bronner & M. Peterlik, eds.) A. Liss, New York: 59-64 (1984).

Nelson, M.T. Interactions of divalent catins with single calcium channels from rat brain synaptosomes. J. Gen. Physiol. 87: 201-222 (1986).

Abstracts:

Nelson, M.T. Permeant ions affect the conductance and open times of single calcium channels from rat brain. J. Physiol. 357: 58P (1984).

Lederer, W.J. and M.T. Nelson. Single Na-K channel current measurements from calf heart muscle. J. Physiol. 357: 46P (1984).

Worley, J.F. Sodium channels in planar lipid bilayers: Effects of divalent cations on ion permeation. Biophys. J. 45: 186a (1984).

Nelson, M.T. Single calcium channel current measurements from brain synaptosomes in planar lipid bilayers. Abst.Soc. Neuroscience. 9: 508 (1983).

Nelson, M.T. Reduction of single calcium channel currents by lanthanum and cadmium. Biophys. J. 45: 395a (1984).

Nelson, M.T., R. Reinhardt, and W.J. Lederer. Sodium channels from mammalian heart muscle incorporated into planar lipid bilayers. Biophys. J. 45: 185a (1984).

Nelson, M.T. and R. Reinhardt. Single ion channel current measurements from rat brain synaptosomes in planar lipid bilayers. Biophys. J. 45: 60-62 (1984).

Each section of this report will consist of a brief summary of one of the above components of the project.

Staff members on this project attended the following scientific meetings to present the experimental results from this project:

R.J. French (invited speaker) XXIXth International Physiological Congress, Sydney, Australia, 8/83.

R.J. French, M.T. Nelson, J.F. Worley. Biophysical Society Meeting, San Antonio, TX, 2/84.

R.J. French (invited speaker) and B.K. Krueger. Gordon Research Conference on "Ion channels in muscle and other excitable membranes", 8/84.

R.J. French (presented plenary paper), B.K. Krueger, J.F. Worley, and M.T. Nelson, invited participants in 4th Biophysical Discussions on Ion Channels, Airlie, VA, 10/83.

M.T. Nelson, 13th Annual Meeting of the Society for Neuroscience, Boston, MA, 11/83.

M.T. Nelson, meeting of the Physiological Society, Cambridge, UK, 7/84.

M.T. Nelson, (invited speaker), Symposium on "Calcium and Phosphate Transport across Biomembranes" Vienna, Austria, 3/84.

J.F. Worley, Attended (with fellowship) Cold Spring Harbor course on "Advanced Electrophysiological Techniques", 7/84.

C. SODIUM CHANNELS IN PLANAR BILAYERS.

During the first year of this contract, voltage-dependent sodium channels from rat brain membranes were incorporated into planar phospholipid bilayer membranes as shown by Krueger et al., 1983 (1). The channels were activated by batrachotoxin (BTX) on the side opposite rat brain membrane addition (trans) and were blocked by saxitoxin (STX) from the side of vesicle addition (cis). This allowed the assignment of the cis side as the outside of the bilayer with respect to the channels. Both macroscopic (multichannel) and single channel currents were studied. The single channel conductance was 30 pS (30×10^{-12} $ohms^{-1}$) in symmetrical 0.5 M NaCl. STX blocked with an apparent dissociation constant of about 4 nM. Stepwise, unitary current fluctuations were observed which were due to the blocking and unblocking of individual sodium channels. Single BTX-activated sodium channels were selective for sodium over potassium, cesium, and chloride. Hyperpolarization favored channel closing. Block of single sodium channels by STX was voltage-dependent with hyperpolarizing potentials favoring block. There is little doubt that these channels are identical to those that are responsible for the depolarizing phase of the action potential of nerve cells and that are the only known site of action of STX and TTX. Details of these results can be found in Krueger et al., 1983 (2). Several particularly interesting findings came out of this work. This was the first report in the literature of the successful incorporation of voltage-dependent sodium channels into an artificial membrane. By taking advantage of the fact that in the presence of BTX, the channels are nearly always open at membrane potentials of ± 60 mV, we demonstrated stepwise, unitary current fluctuations due to the blocking and unblocking of individual sodium channels by STX. This demonstrated for the

first time that block by STX is all-or-none, that is, a channel is either fully open or completely blocked by STX. Another interesting result that is described in more detail in French et al., 1984 (3) is that block by STX is affected by the membrane potential with depolarizing potentials reducing the potency of block by up to 50-fold.

All experiments on sodium channels reported here have been carried out with channels incorporated into planar bilayers formed from solutions of phospholipids (either phosphatidylethanolamine or phosphatidylethanolamine and phosphatidylserine) in decane as described in Krueger et al., 1983 (2). Membrane vesicles, prepared from homogenates of rat brain, are added to one side of the planar bilayer (the cis side) and channel incorporation monitored by observing stepwise single channel current fluctuations with a potential applied to the membrane. Unless otherwise specified, the solutions on both sides of the planar bilayer contained identical electrolyte compositions (usually 500 mM or 250 mM NaCl) and batrachotoxin (BTX) was present on the side opposite vesicle addition (the trans side). Ionic currents through single channels were recorded using either a Yale Mark IV patch clamp or one of several homemade devices constructed in our electronics shop. With the membranes used in these studies (normally 0.25 mm in diameter), we can record current steps of about 1 pA at 500 Hz or about 0.2 pA at about 100 Hz. These bandwidths place limitations on the resolution of fast gating events and we are working on methodologies to reduce the membrane size in order to increase the effective recording bandwidth.

D. VOLTAGE-DEPENDENT BLOCK OF SODIUM CHANNELS BY SAXITOXIN AND TETRODOTOXIN.

Summary. Our preliminary finding (2), that the potency of block of sodium channels by STX varied with the membrane potential has now been investigated in detail. All experiments were conducted on bilayers containing only one or a few sodium channels. The voltage dependence of STX block was evaluated from steady state measurements of the fractional block as a function of STX concentration over a range of membrane potentials. It was found that the dissociation constant (K_I) for STX block ranged from about 1 nM at -60 mV (near the normal resting potential) to about 50 nM at +60 mV. By evaluating the kinetic parameters of unitary single channel fluctuations induced by STX, we were able to determine that depolarization decreased the blocking rate constant and increased the unblocking rate constant. At each potential, the K_I derived from kinetic parameters agreed well with the K_I obtained from steady state determinations.

Voltage-dependent STX block was entirely unexpected. Several investigators had previously considered the possibility that STX or TTX block was voltage-dependent (2,4-6) but in each case no evidence could be found to demonstrate such an effect of voltage on block. One report (7) suggested that depolarization of heart cells increased the potency of TTX block, however, the validity of the experimental methods used to carry out those experiments have been questioned (8), and more recent studies have failed to confirm that finding (9). As summarized above, our results demonstrate that depolarization reduces the potency of STX block at least under the specific experimental conditions used in our experiments.

A voltage-dependent process suggests that one or more electric charges are

interacting with the membrane electric field. The most likely candidate for those charges is the STX molecule itself which is a divalent cation under physiological conditions. One possible mechanism is that the binding site for STX lies inside the channel at a location within the membrane electric field. Thus block by STX, approaching from the outside, would be favored by negative-inside (hyperpolarizing) potentials, making the potency of block greater (10,11). Our initial results appeared to be consistent with this model. However, we have subsequently compared the action of TTX, a monovalent cation, with the divalent STX. The K_I's for these two toxins vary identically with voltage, changing e-fold for a 40 mV change in voltage (Figure 1). Since the two charges on STX are separated by only 3-4 A, it is highly unlikely that one of these charges could penetrate into the transmembrane electric field without the other. Given identical effects of voltage on the action of the toxins with charges of +1 and +2, the voltage-dependence is unlikely to result from entry of the toxin molecules into the transmembrane field. The voltage dependence must arise from some common, voltage-dependent step in the binding and blocking reactions, perhaps a voltage-dependent conformational change in the channel protein itself.

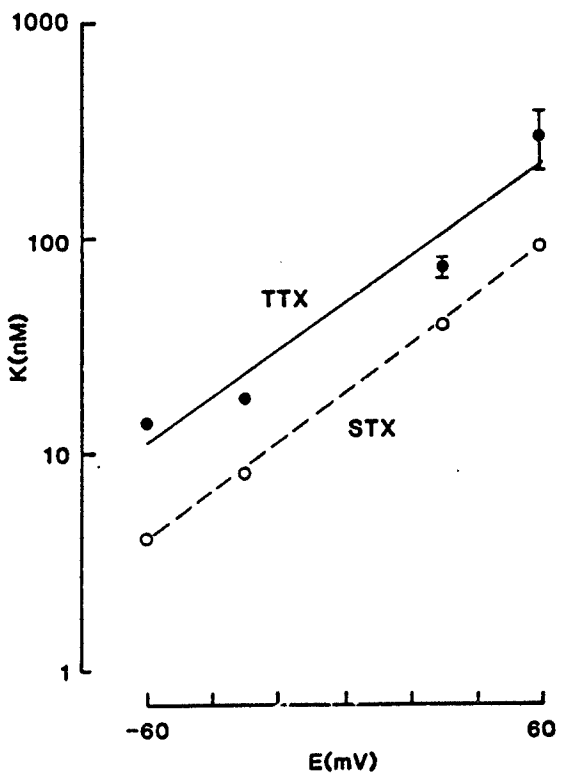

Figure 1. Apparent dissociation constants for sodium channel block by STX and TTX show identical depedence on voltage.

E. DIVALENT ION EFFECTS ON SODIUM CHANNEL CURRENTS.

Block of the ion permeation pathway of sodium channels by divalent cations can be attributed to two main factors. The first is that divalent cations can bind to, or screen charges located on the membrane surface thereby producing alterations in the membrane surface charge. Secondly, a direct, more specfic interaction between the Na channel protein and the divalent cation blocker may also occur. Both factors, either separately or in concert, could influence the movement of sodium ions through the sodium channels.

Figure 2 illustrates the variation of the single channel conductance as the sodium ion concentration on both sides of the bilayer is altered; in each case the divalent ion concentration is <100 nM. Sodium channels were incorporated into phosphatidylethanolamine membranes which have no net charge so that the effects of surface charge can be eliminated. The single channel conductance saturates at a maximum value of 32 pS and the half maximal conductance occurs at 37 mM sodium. This saturation behavior which is analogous to Michaelis-Menten enzyme kinetics, provides evidence that the permeant ion interacts with a site as it passes through the channel. In negatively charged phosphatidylserine membranes, where the surface charge density is high, the conductance saturates at 32 pS and the dissociation constant for sodium is 28 mM (data not shown). Therefore surface charge on the membrane lipid appears to play only a minor role in the ion permeation process.

The direct effect of divalent cations on the movement of sodium through sodium channels into neutral membranes is shown in figure 3. The single channel current is plotted as a function of the applied potential. The closed circles show data in which the solutions on both sides of the bilayer contained 125 mM sodium and the divalent ion concentration is <100 nM. The open circles and squares represent the single channel currents after symmetical addition of 20 mM strontium or 10 mM calcium, respectively. For both divalent cations there is a reduction in the single channel current in a voltage-dependent manner, consistant with a site of interaction located approximately 20-23% of the electrical distance from the extracellular side. The outward currents were reduced in a voltage-independent manner. Calcium appears to have a higher affinity than strontium for these sites as indicated by the greater reduction of the single channel currents in the presence of calcium. When these experiments were performed on sodium channels incorporated into negatively charged membranes, virtually identical results were obtained, again indicating little effect of lipid surface charge on the ion permeation process. Similar results were also obtained with barium, magnesium, and manganese. The order of efficacy is different from the affinity sequences for binding of these divalent cations to phospholipids. These results taken together are inconsistent with a non-specfic screening effect by the divalent cations and strongly suggest a direct interaction between the divalent cations and a site in the sodium channel protein.

Competition between sodium and the divalent blocker is shown in figure 4. Sodium channels were incorporated into neutral membranes and the solution compositions were changed as indicated. The double reciprocal plot illustrates that strontium increases the apparent K_m for sodium from 37 mM to 400 mM as shown by the shift in the X-intercept. The maximum conductance is not substantially altered as indicated by the small change in the Y-intercept. Identical results are obtained by the symmetrical addition of 10 mM calcium except that the K_m for sodium is substantially increased, reflecting a higher affinity for calcium. These observations suggest competition between sodium and the divalent cations

for a site or sites located in or near the permeation pathway.

In addition to the competitive interactions between sodium and divalent cations, competitive interactions exist between the divalents and STX. Addition of triethyloxonium tetrafluoroborate (TMO), a carboxyl modifiying reagent known to remove STX binding, eliminates STX block at the single channel level, reduces the single channel conductance between 20-30%, and reduces the ability of calcium to influence sodium ion movement. Calcium also increases the K_d for STX block and binding by 2-3 fold, suggesting competition between calcium and STX. These results suggest a multisite interaction between the divalent cation blocker and the sodium channel protein. One of these sites is also responsible for STX binding/blocking which can be removed by TMO modification and can influence sodium ion movement through sodium channels.

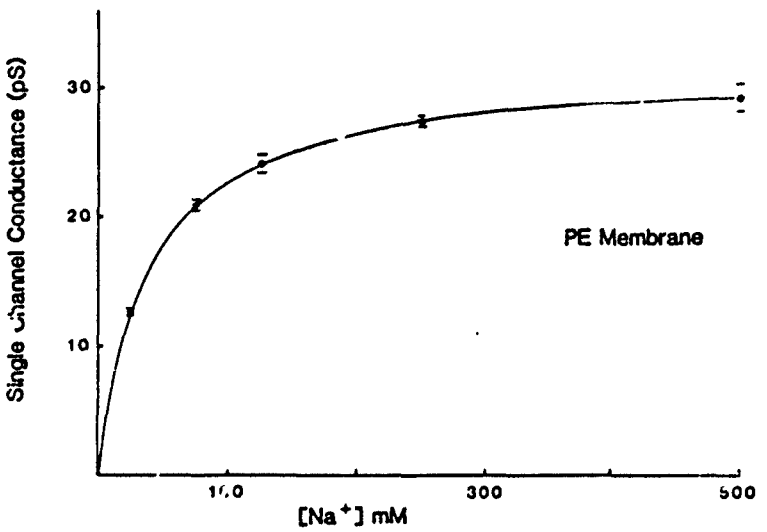

Figure 2. Conductance of a single sodium channel is a saturating function of sodium concentration, suggesting that only one sodium ion can be present in the channel at one time.

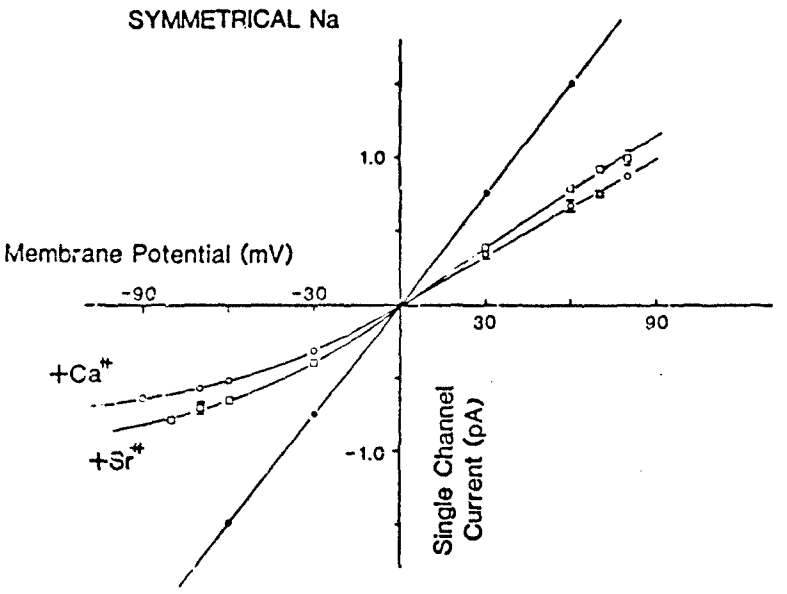

Figure 3. The divalent ions, calcium and strontium, cause a voltage-dependent reduction of single channel currents. Block of inward currents is both greater and more voltage-dependent than block of outward currents, even though the divalent ions were added symmetrically.

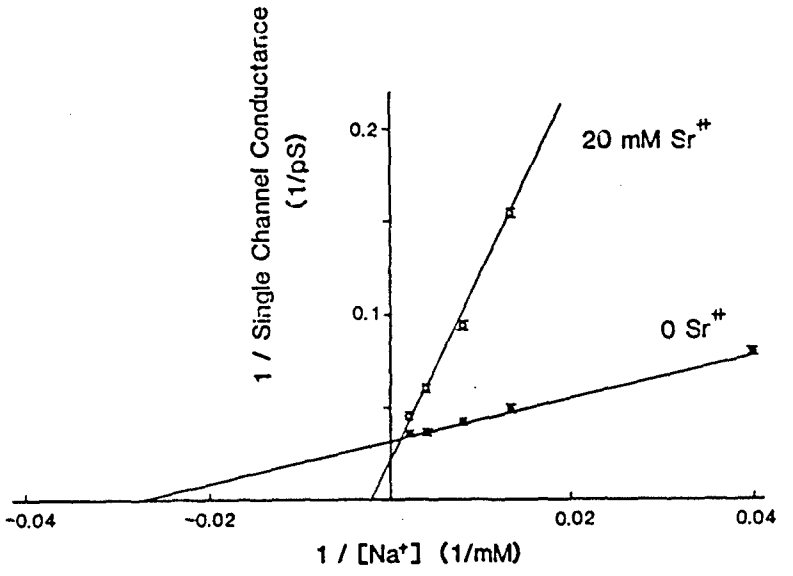

Figure 4. Reciprocal plots showing the dependence of unit conductance of sodium channels on sodium concentration, in the presence and absence of strontium, suggest that sodium ions compete with strontium for the blocking site.

F. CALCIUM CHANNELS FROM RAT BRAIN IN PLANAR LIPID BILAYERS.

Voltage-dependent calcium channels from rat brain were incorporated into planar lipid bilayers (Nelson, French, Krueger, 1984 (see ref. 12)). A membrane fraction from rat brain median eminence was used as a source of these channels because that region of the brain consists primarily of nerve terminals. Nerve terminals are thought to have a high concentration of calcium channels. These brain single calcium channels have the following properties:

1. Selectivity: These channels select for calcium, barium, strontium, manganese over sodium, potassium, chloride, lanthanum, cadmium.
2. Unit Conductance: Barium (8-9 pS) > Calcium, Strontium (5-6 pS) > Manganese (3-4 pS) (Nelson et al., 1984; Nelson, 1934).
3. Voltage-dependence: Membrane depolarization increased the opening rate constant and decreased the closing rate constant (12). The relationship between the probability of the channel being open and voltage could be described by the Boltzmann equation with an "apparent gating charge" of 1.5-2.0 (13).
4. Ion-dependence of kinetics: It has been traditionally thought that the kinetics of an ion channel are independent of the nature of the ion carrying charge through the channel. A significant finding of our studies was that the nature of the permeant divalent cation not only affected the single channel conductance, but it also affected the channel's closing rate constant (i.e. mean open times), with the same order of potency, i.e. barium > calcium, strontium > manganese (Figure 5). This observation suggests that competition by the permeant ions for common sites in the channel may regulate both the rate of ion passage through the channel and it's closing rate (i.e. occupancy of a site in the channel by a permeant ion would impede channel closing). This suggestion is further supported by the observation that the addition of manganese to solution containing a more permeant ion both decreased the single channel conductance and closing rate constant (Nelson, 1984).
5. Block of channel by inorganic calcium channel blockers: A variety of inorganic ions including cadmium, cobalt, nickel, manganese, and lanthanum can reduce calcium currents in many preparations. We found that these inorganic calcium channel blockers reduce the single channel current in a dose dependent manner, with the order of potency being lanthanum > cadmium > manganese (Figure 6; Nelson et al., 1984). The efficacy of block depended not only on the nature of the blocking ion but also the nature of the permeant ion. The block was consistent with one blocking ion binding to a site in the channel and thereby impeding ion flow through the channel.

G. CARDIAC ION CHANNELS

To determine if "biological" channels from heart muscle sarcolemma can be incorporated into bilayers, batrachotoxin (BTX)-activated, tetrodotoxin (TTX)-sensitive Na channels were first identified (Fig. 7). An interesting difference between heart Na channels and brain Na channels in planar lipid bilayers under identical conditions is that brain Na channel has a greater single channel conductance (30 pS) than the cardiac Na channel (19 pS). In addition, a voltage-dependent, rectifying channel that permits both Na and K to permeate but not Cl has been identified (Fig. 8). Membrane hyperpolarization increases both the probability of the channel being open and the amount of current that can flow through the open channel. This channel is not affected by removing calcium or by TTX and may find a role as the pacemaker current as described by DiFrancesco (14). Identification of known cardiac ion channels in the bilayer increases the confidence that it may also be possible to incorporate cardiac

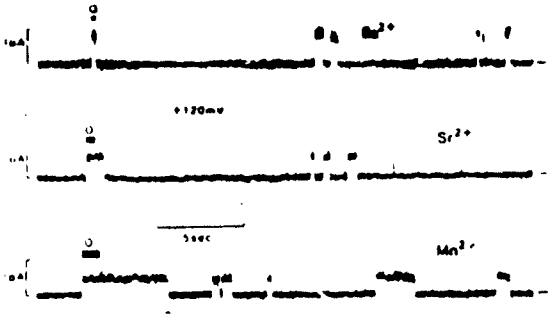

Figure 5: Ion-dependence of single calcium channel currents and open times. Solid bars, labeled O, indicate the mean open times.

Figure 6: Cadmium block of single calcium channel currents. Solid bar on the left of each record indicates the current level where the channel is closed.

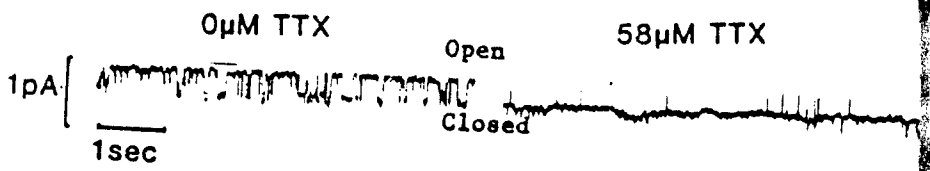

Figure 7: TTX block of BTX-activated Na channel from heart.

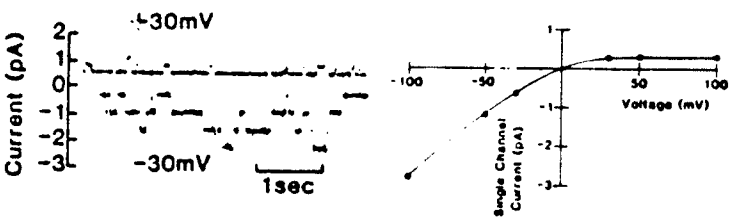

Figure 8: Voltage-dependant, rectifying Na-K channel from heart. Solution (mM): cis side 250 NaCl, 25 KCl; trans side 250 KCl, 25 NaCl.

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